International Journal of Probiotics and Prebiotics Vol. 7, No. 3/4, pp. 135-144, 2012
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DETECTION OF LACTOBACILLI IN MONTHLY MAIL-IN STOOL SAMPLES FROM 3-18 MONTHS OLD INFANTS AT GENETIC RISK FOR TYPE 1 DIABETES

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[Received July 12, 2011; Accepted July 25, 2012]

ABSTRACT: ***The feasibility to detect lactobacilli in mail-in infant stools collected monthly from 3-18 months old children was investigated. The aim was to determine total lactobacilli and Lactobacillus plantarum (L. plantarum) content (ng/g feces) in 50 infants each from Colorado (648 samples), Finland (624 samples) and Sweden (685 samples) who participated in the TEDDY (The Environmental Determinants of Diabetes in the Young) study. Total lactobacilli content varied markedly between 5 and 16,800 ng/g feces in the three clinical sites within and between individuals especially in infants. L.plantarum also varied markedly intra- and inter-individually from <0.5 - 736 ng/g feces. A higher variability of total lactobacilli was found before 10 months of age than after in the three different clinical sites. Sweden had the lowest total lactobacilli content compared to Colorado and Finland while the L.plantarum content was higher in Sweden. Mail-in stool samples from infants should prove useful in analyzing probiotics in childhood.***

KEY WORDS: Autoimmune Diabetes, Celiac Disease, Infants, Microflora, Probiotics

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INTRODUCTION

The intestinal microflora is thought to be important for the development of the immune system in early infancy and may contribute to the wellbeing and health of children (Walter, et al., 2001). *Lactobacillus,* a genus of Gram-positive, anaerobic or micro-aerophilic heterogeneous lactic acid producing bacteria are common in the intestinal microflora of healthy adults (Heilig, et al., 2002). *Lactobacillus* may grow at low pH and by its production of lactic acid they often inhibit pathogenic bacteria or other harmful microbes from colonization and growth. Neonates acquire lactobacilli by oral exposure to vaginal lactobacilli during delivery. The early microflora in newborn infants may therefore differ by the mode of delivery (Biasucci, et al., 2010, Nelun Barfod, et al., 2011). Infants born by caesarean section harbor bacterial communities similar to those found on the skin surface (Dominguez-Bello, et al., 2010).

Lactobacillus is commonly detected in stools of infants (Mackie, et al., 1999, Ahrne, et al., 2005, Dominguez-Bello, et al., 2010) and was reported in stool samples of neonates already within 24 h after birth (Dominguez-Bello, et al., 2010). Lactobacilli content or colonization rates in early infant stools (1-12 months of age) varies between and within the infant by age, mode of delivery, geographical area and the food source of the infant such as breast milk or formula in the first months (Balmer and Wharton. 1989, Gronlund, et al., 1999, Ahrne, et al., 2005, Vael, et al., 2011). Earlier studies with bacterial plating and culture showed defined quantities (10^{7-9} CFU/g feces) of *Lactobacillus* in infant stool recently also confirmed by polymerase chain reaction (PCR) and sequence analysis (Hall, et al., 1990, Kleessen, et al., 1995, Haarman and Knol. 2006).

The World Health Organization (WHO) defines probiotics as living microorganisms which when administered in adequate amounts confer a health benefit on the host

and several *Lactobacillus* species are common probiotic supplements and products (FAO/WHO. 2006). It has been reported that lactobacilli may be beneficial to autoimmune diseases such as inflammatory bowel disease (IBD) (Lieske, et al., 2005, Schmidt, et al., 2010), celiac disease (Lindfors, et al., 2008), type 1 diabetes (T1D) (Roesch, et al., 2009, Petrovsky. 2010) and multiple sclerosis (MS) (Lavasani, et al., 2010). The etiology and pathogenesis of these organ-specific autoimmune diseases are not fully understood. Both type 1 diabetes and celiac disease have long subclinical asymptomatic prodromal disease states defined by the presence of autoantibodies against target autoantigen (Knip, et al., 2005, Dib and Gomes. 2009). It cannot be excluded that exposure to lactobacilli during the prodrome may affect disease pathogenesis. However, little is known about the relationship between colonization and consumption of probiotics during infancy.

The Environmental Determinants of Diabetes in the Young (TEDDY) study is a consortium comprising six clinical centers in Europe and the USA, which has already from 3 months of age enrolled children at increased genetic risk for type 1 diabetes and celiac disease (TEDDY Study Group. 2007, Hagopian, et al., 2011). The main goals of the multicenter and multinational consortium are to identify environmental factors and gene environment interactions causing islet autoimmunity as defined by persistent autoantibodies against GAD65, IA-2 or insulin and T1D (TEDDY Study Group. 2007). As part of the study, the parents were asked to mail stool samples on a monthly basis. These mail-in stools samples have been obtained to be used in future nested case-control studies to test hypotheses related to the appearance of islet or celiac disease autoantibodies (TEDDY Study Group. 2007). Here we test a subset of samples to evaluate the present approach to collect, store and process stool samples. It was hypothesized that mail-in stool samples at ambient temperatures were comparable to previous more rigorous approaches (Hall, et al., 1990, Kleessen, et al., 1995, Gronlund, et al., 1999, Ahrne, et al., 2005, Haarman and Knol. 2006) in content of total lactobacilli and *L.plantarum*, in stool samples collected at 3-18 months of age.

SUBJECTS AND METHODS

Subjects. In the TEDDY study, 420 000 newborn infants were screened for T1D and celiac disease high-risk HLA genotypes in six different clinical centers (Europe: Finland (FIN), Germany, Sweden (SWE) and USA: Colorado (COL), Georgia and Washington) (TEDDY Study Group. 2007, Hagopian, et al., 2011). The subjects in this study were 50 infants each from COL, FIN and SWE (Table 1). Their parents mailed stool samples on a monthly basis at the age of 3-18 months resulting in a total of 648 stool samples from COL, 624 stool samples from FIN and 685 stool samples from SWE (Table 2). The infants were healthy and were selected because they were negative for islet and tissue transglutaminase autoantibodies. The distribution of the T1D and celiac disease high-risk HLA genotypes were DQ2/8 (n=62), DQ8/8 (n=24) and DQ2/2 (n=14), the distribution between COL, FIN and SWE was comparable.

TABLE 1. Distribution of participating infants by country, gender, age, HLA genotype and mode of delivery.

	COLORADO	FINLAND	SWEDEN
Gender			
Female	29	28	20
Male	21	22	30
Total	50	50	50
HLA genotype	28	34	27
DQ 2/8	12	12	14
DQ 8/8	10	4	9
DQ 2/2	50	50	50
Total			
Mode of delivery			
Unknown	1	1	3
Vaginal			
Normal vaginal	35	37	43
Instruments plus Vaginal	2	2	1
Subtotal	37	39	44
Caesarean section	12	10	5
Total	50	50	50

Stool sample processing. The stool samples from the different clinical centers were mailed to the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) repository in Germantown, MD for COL, to the respective TEDDY clinic in Turku, Tampere or Oulu for FIN or to the TEDDY clinic in Malmoe for SWE. All samples were kept at -80° C and shipped on dry ice from FIN to the NIDDK repository. All samples from the 150 infants were sent on dry ice to Tampere, Finland for processing. Each stool sample was weighed to determine wet weight in grams, cut and suspended in phosphate-buffered saline (PBS) to prepare a 10% (w/v) suspension. A 0.5 mL aliquot was shipped on dry ice to Malmo, Sweden for DNA extraction and analysis of lactobacilli by PCR.

Extraction of stool sample DNA: Stool sample suspensions were stored at -80° C until analysis within 2-4 months. DNA was extracted with QIAamp DNA stool Mini Kit (Qiagen) according to the manufacturer's instructions. The quantity and purity of the extracted DNA was checked by a NanoDrop 1000 Spectrophotometer instrument (Thermo Scientific). The

TABLE 2. Total lactobacilli in ng DNA/g faeces are shown for each month of sampling. Number of stool samples submitted per month from TEDDY children in Colorado, Finland and Sweden respectively.

Age	Colorado	Total lactobacilli		Finland	Total lactobacilli		Sweden	Total lactobacilli	
Months	n	Median	Range	n	Median	Range	n	Median	Range
3	50	134	6-7080	50	113	10- 4568	50	40	3- 16800
4	40	140	5-7024	47	134	24-14676	47	47	4-3128
5	49	177	5-16800	47	165	7-5280	49	61	5-944
6	39	175	18-5144	45	170	10-4320	45	59	6-7432
7	46	171	3-16800	46	269	8-7872	45	60	7-4432
8	47	172	10-8240	45	193	11-16800	50	54	8-2568
9	42	172	14-16240	38	131	11-6704	46	51	9-7920
10	48	136	11-3272	42	112	25-2584	46	48	10-109
11	46	109	7-494	36	133	15-8320	45	55	10-1208
12	42	121	26-454	38	104	5-2072	45	54	8-147
13	44	118	22-896	40	121	30-976	45	54	8-677
14	37	104	13-727	39	116	20-15440	45	52	14-438
15	41	110	15-462	34	100	37-1520	44	57	15-770
16	33	98	10-551	35	122	36-5928	43	50	9-258
17	23	98	15-540	31	129	36-1120	32	56	20-758
18	10	87	12-184	11	117	28-1320	8	59	39-141
Mean		133			139			57	
Total	648			624			685		

DNA was then stored at -20° C until further DNA analysis.

Detection of total Lactobacilli and *L.plantarum* in stool: Stool lactobacilli and *L.plantarum* content was determined in duplicate samples by quantitative PCR (q-PCR) using RealPlex^2 (Eppendorf) instrument as previously described (Haarman and Knol. 2006, Berggren, et al., 2011). The qPCR was carried out at the accredited laboratory of Probi AB, Lund, Sweden. Total lactobacilli content was determined by analyzing the presence of a conserved 16S ribosomal RNA sequence in 20 µl PCR amplification reaction mixture containing 10 µl Platinum SYBR green qPCR superMix-UDG (Invitrogen), 1 µl 0.1µM of forward (5'-AGCAGTAGGGAATCTTCCA-3') and reverse (5'-CACCGCTACACATGGAG-3') primers (Applied Biosystems), 2 µl stool DNA and 7 µl RNase-free DPCE-treated H_2O (Haarman and Knol. 2006, Berggren, et al., 2011). The temperature profile of the q-PCR consisted 2 min at 50°C, 2 min at 95°C, followed by 40 cycles of 15 s at 95 °C, 30 s at 61 °C and 30 s at 72°C. Species specific primers and probe targeted on the 16S intergenic spacer region were used to detect *L.plantarum* with q-PCR (Berggren, et al., 2011). The q-PCR assays were performed in 25 µl PCR amplification mix containing 12,5 µl Platinum Q-PCR Super Mix-UDG (Invitrogen), 0,2 µM (0,1125 µl) of both primers (FW5'-CGGTGTTCTCGGTTTCATTATG-3' and REV 5'-CCTACACACTCGTCGAAACTTTGT-3') (Applied Biosystems), 100 µM

Taqmanprobe6-Fam(5'-CTTGTTCTTTGAAAACTAG3'-MBG) (Applied Biosystem) and 5 µl stool DNA. *L.plantarum* DNA extracted from pure cultures was used as standard (Tannock. 1999, Haarman and Knol. 2006, Berggren, et al., 2011). The final concentration of total lactobacilli and L.plantarum were given as ng DNA/g feces. The detection limit for total lactobacilli was 1 ng DNA/g feces and 0.6 ng DNA /g feces for *L. plantarum.*

Statistical evaluation: Box and whiskers plots or line diagram of individual samples were used to display the data. Tests for normality failed for content of *L.plantarum* and total *Lactobacillus*. Mann Whitney U test and Wilcoxon signed-rank test were used to compare levels. A two tailed p value of <0.05 was considered significant. GraphPad was used to calculate AUC. The statistical package used was SPSS 18.

RESULTS

DNA content: The concentration of DNA extracted in a total of 1,957 stool samples varied from undetectable (4 samples; 0.2 %) to 1,647 µg/g feces (Figure 1). The results of the 648 stool samples from COL (Figure 1, Panel A) , 624 stool

samples from FIN (Figure 1, Panel B) and 685 stool samples from SWE (Figure 1, panel C) showed comparable median values. The content of DNA extracted from stool samples mailed during the winter (January, February, September, October, November and December) was not different from summer (March, April, May, June, July, August) in COL (Figure 2, panel A) and SWE (Figure 2, panel C), while FIN showed higher content in the winter than in summer (Figure 2, panel B).

FIGURE 1. The total DNA recovery varies in panel A, COL; panel B, FIN and panel C, SWE. Similar variation of the DNA recovery can be seen between the countries.

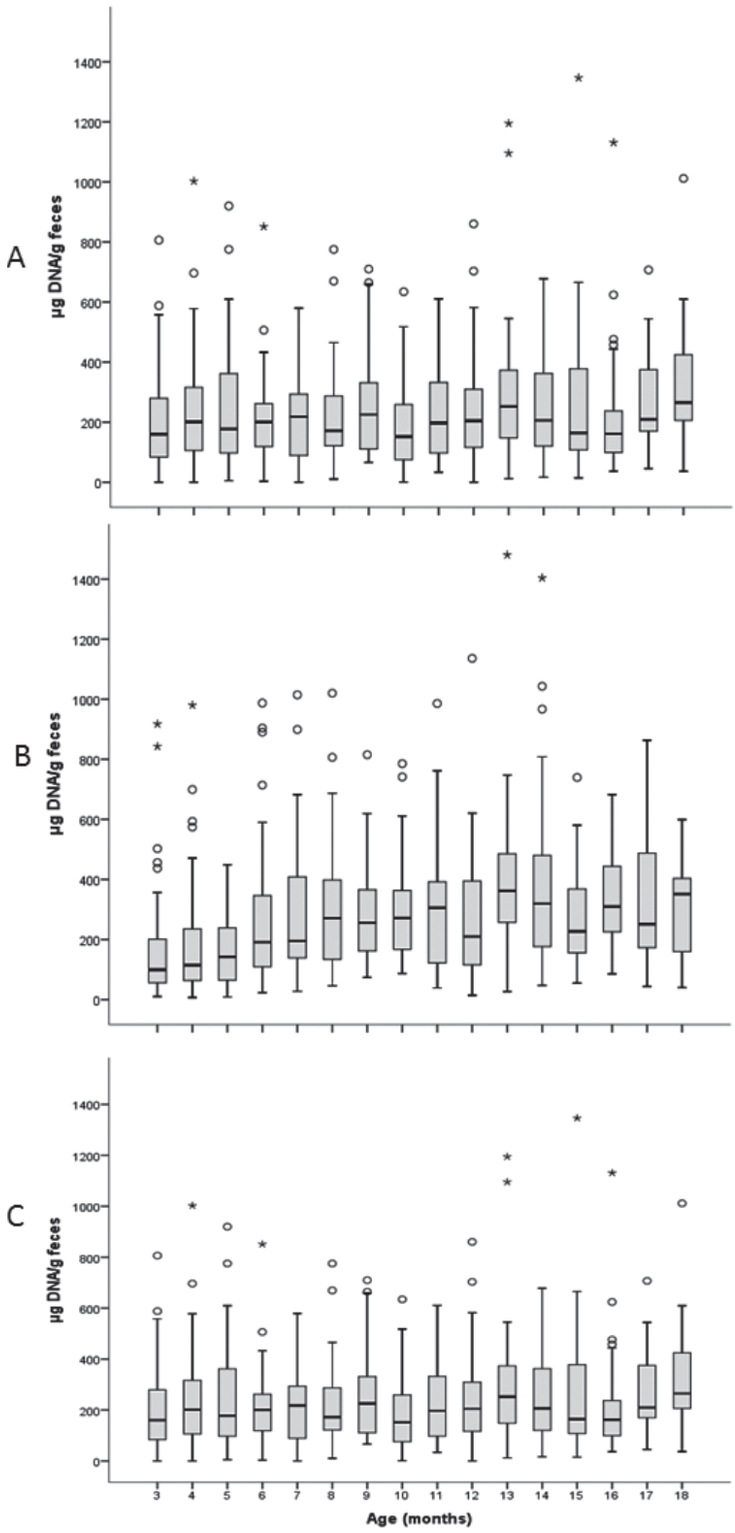


FIGURE 2. DNA content (microgram DNA/g feces) obtained in mail-in samples from 50 children each during four calendar years in COL (panel A), FIN (panel B) and SWE (panel C). There was no seasonal variation in COL and SWE while the DNA content was higher in FIN during the winter (p=0.03).

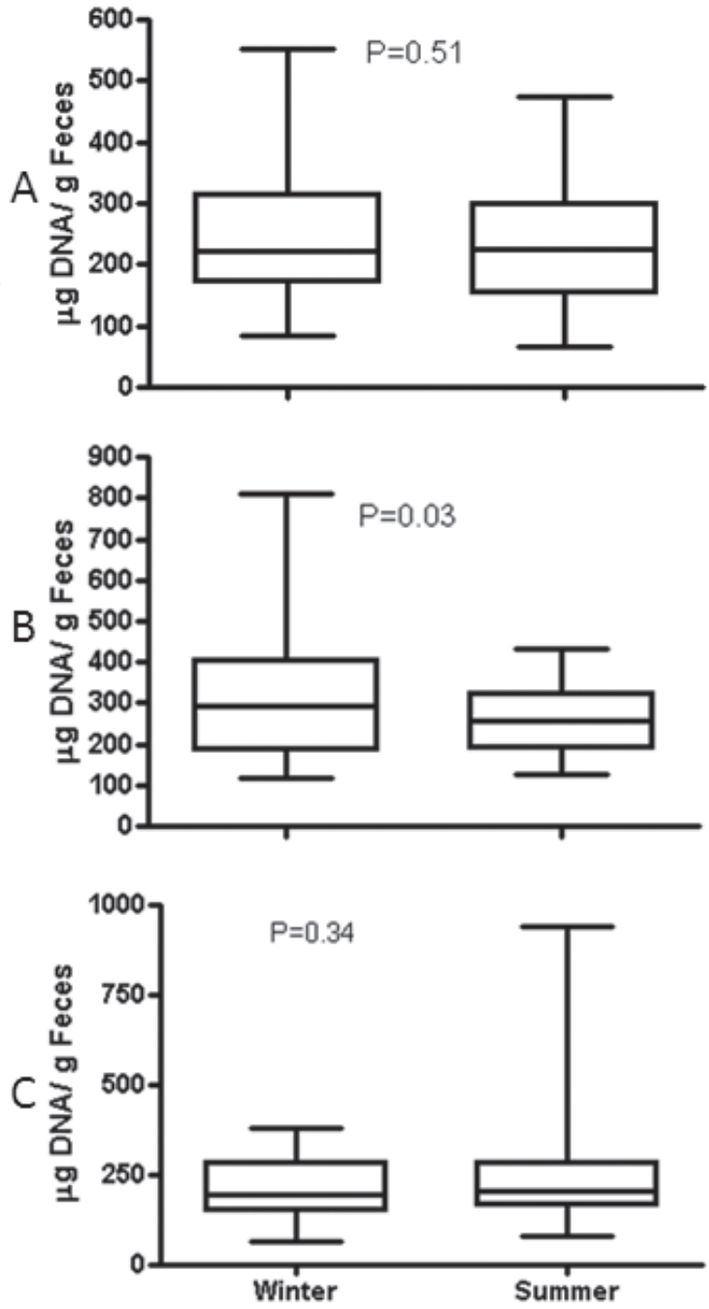
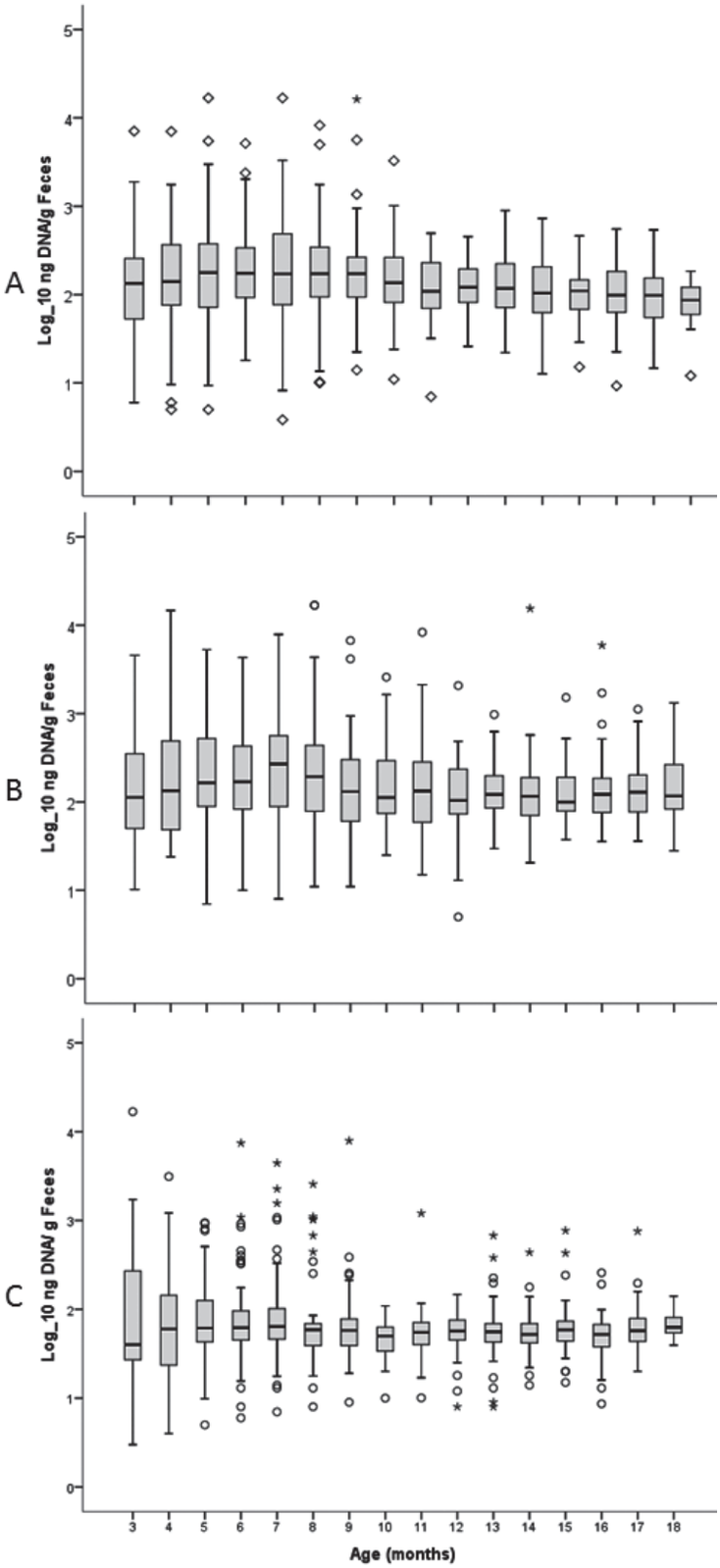


FIGURE 3. Box and whiskers plots of total lactobacilli content (ng DNA/g feces) in all infants at the age of 3-18 months in COL (panel A), FIN (panel B) and SWE (panel C).



Total lactobacilli: The total lactobacilli content varied between 5 – 16,800 ng lactobacilli/g feces in both COL (Figure 3, panel A), FIN (Figure 3, panel B) and SWE (Figure 3, panel C). Total lactobacilli content for each child was computed from the area under the curve (AUC) of each peak multiplied by the number of peaks (Figure 4). While FIN and COL did not differ, the total AUC was lower in SWE compared to both COL (P=0.0001) and FIN (P=0.001). The box plot with whiskers analyses (Figure 3) indicted increased variability at lower age. It was found that the total AUC was increased in samples from 3-10 months old compared to 11-18 months old children in COL (P=0.0001), FIN (P=0.001) and SWE (P=0.0001)(Figure 4, panel B).

FIGURE 4. Panel A. Lactobacilli content (ng DNA/g feces) shown as the sum of AUC of total lactobacilli in samples from 3-18 months of age in 50 children each from COL, FIN and SWE, respectively. The box plot with whiskers analysis shows that the AUC content was higher in FIN and COL compared to SWE. **Panel B. Total lactobacilli AUC comparing the 3-10 with 11-18 months samples between COL, FIN and SWE.** The box plot with whiskers analysis shows that the AUC content was higher at 3-10 compared to 11-18 months samples at all three sites.

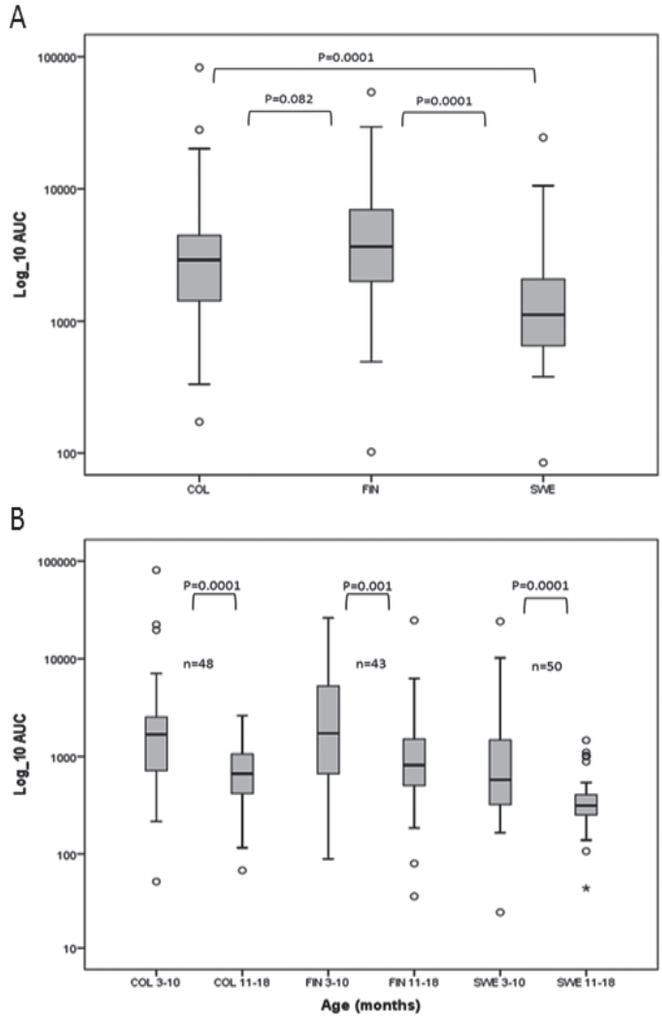
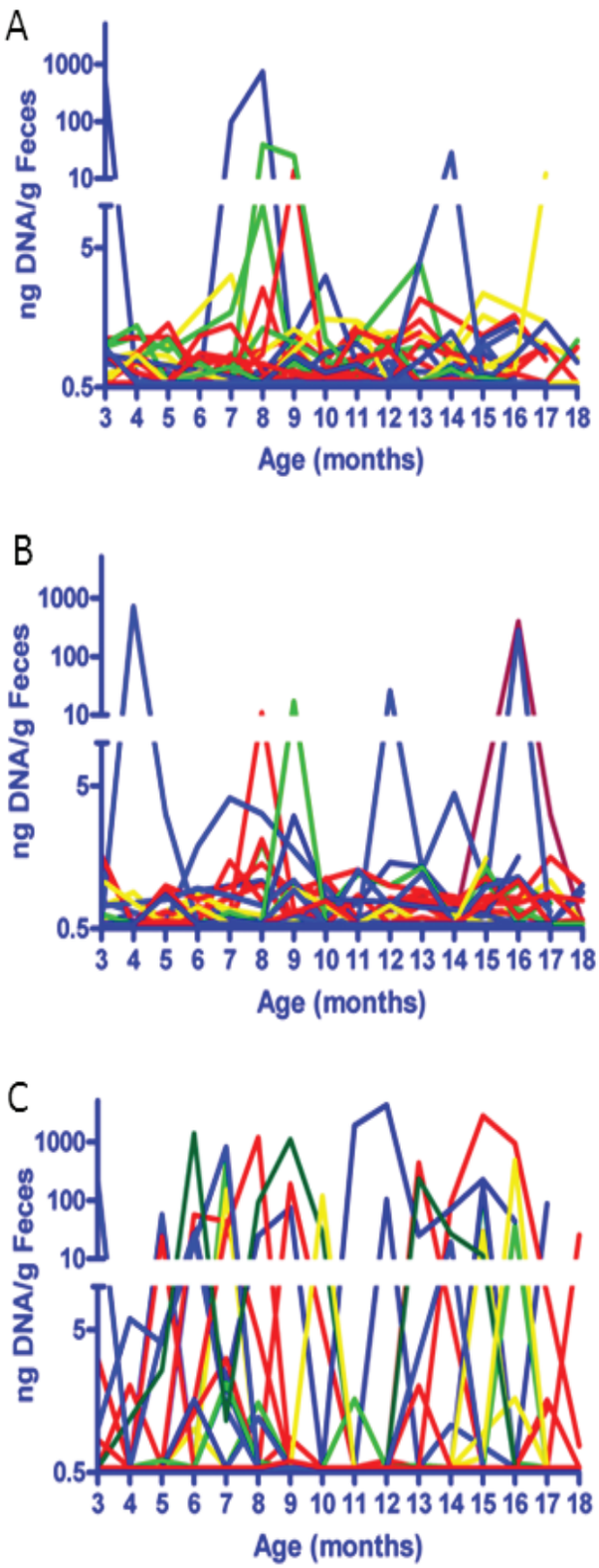


FIGURE 5. *L. plantarum* content (ng DNA/g faeces) shown individually for all 50 infants at the age of 3-18 months in COL (A), FIN (B) and SWE (C). Significant excursions in *L. plantarum* content were evident at all three sites.



***L. plantarum*:** The *L.plantarum* content varied markedly between 0.5-1,000 ng *L.plantarum*/g feces both within and between individuals in COL, FIN and SWE (Figure 5). As the excursions in *L. plantarum* varied markedly results are presented on a log scale for 36 children in COL (Figure 5A), 32 children in FIN (Figure 5B) and 31 children in SWE (Figure 5C) who had a content of *L. plantarum* above the lower detection limit of 0.6 ng/g feces in a single sample. Each line represents an individual infant. Variability and excursions in content was observed in all three clinical sites. The number of peaks for the children was different between the sites (Table 3). It was noticed that as many as 14-19 children had no *L. Plant arum* peaks. The largest number of peaks was found in FIN and COL. The length of each peak also varied between sites as FIN and COL had peak lengths longer than 4 months more often than in SWE (Table 3). Total AUC analysis of the children with peaks (about 36-39% of the children had no peaks similar in COL, FIN and SWE) for *L. plantarum* showed that SWE had higher total AUC compared to both COL (P=0.001) and FIN (P=0.002).

The mode of delivery to these children was known (Table 1), however, our study of only 150 children precluded a meaningful statistical analysis of a possible relationship between total lactobacilli or *L. plantarum* and the mode of delivery.

FIGURE 6. *L. plantarum* content (ng DNA/g feces) shown as the sum of AUC of *L. plantarum* in samples from 3-18 months of age in 50 children each from COL, FIN and SWE, respectively. The box plot with whiskers analysis shows that the AUC content was higher in SWE compared to both COL and FIN, which did not differ.

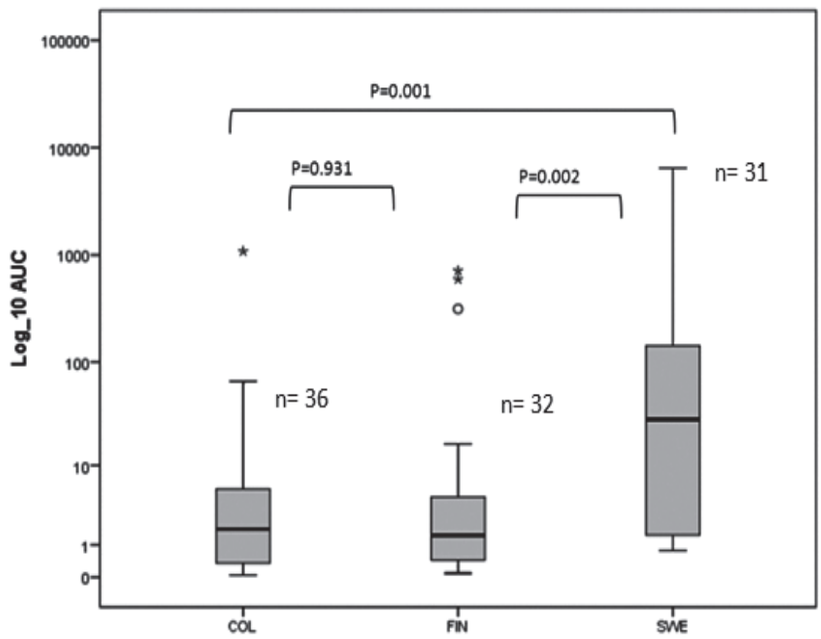


TABLE 3. Number of *L. plantarum* peaks and peak lengths in months in stool samples from TEDDY children.

Number of peaks	0	1	2-5	>5	n
COLORADO	14	7	16	13	50
FINLAND	18	10	10	12	50
SWEDEN	19	15	12	4	50
Peak length in months	1	2	3	4	>4
COLORADO	47	15	10	5	6
FINLAND	42	10	8	2	9
SWEDEN	37	0	3	3	3

DISCUSSION

The major findings in this study of a total of 1,957 mail-in stool samples from 50 children each from COL FIN and SWE, respectively, were the large significant variability of total lactobacilli content and *L.plantarum* content within and between the subjects and also between the three clinical centers. The quality of the stool samples allowed detection of DNA in all the 1,957 samples. The similar DNA content in the samples from the three clinical sites suggested that mail-in stool samples would allow country comparisons. The effect of season was minor. It was therefore possible to conclude that the inter-individual variability of total lactobacilli was comparable between COL, FIN and SWE and there from obtain the results of significantly lower total lactobacilli content in SWE. The variability of total lactobacilli content significantly decreased after 10 months of age (figure 4). The age-dependent variability in total lactobacilli was not reflected in the content of *L. plantarum* as both the inter- and intra-individual variability of this particular strain was apparent at any age (Figure 5). The total lactobacilli exposure was lower in SWE compared to COL and FIN.

In some children the excursions in *L. plantarum* content varied by several orders of magnitude usually but not always in sequential samples. It should therefore be possible to detect the use of probiotics containing *L. plantarum* in future studies of mail-in stool samples. The total *L.plantarum* exposure was higher in SWE compared to COL and FIN. This may be due to a higher consumption of probiotic products or supplements containing *L.plantarum* among children or women in SWE. However, it will be important in future TEDDY studies to analyze ratios between Lactobacilli and *L. plantarum* in the microbiome, as it cannot be excluded that *L. plantarum* is part of, and changes with, the normal gut flora.

The approach to collect the stool samples at the three clinical sites was somewhat different (TEDDY Study Group. 2007). Despite the variability in the way the samples were handled and mailed by ordinary mail in COL, FIN and SWE, the results of both extractable DNA as well as total lactobacilli and *L. plantarum* was largely comparable. The higher DNA content per gram frozen stool in FIN winter samples compared to summer samples would be consistent with the possibility that FIN stool samples were slightly better preserved during the winter months.

The present analysis of 1,957 stool samples from 150 infants from COL, FIN and SWE was selected to evaluate the present approach to collect, store and process stool samples in order to detect and analyze both total lactobacilli as well as specific probiotic species for further analyses. The primary objective of the TEDDY study is the identification of infectious agents, dietary factors, or other environmental exposures that are associated with increased risk of islet autoimmunity and T1D as well as the risk for tissue transglutaminase autoantibodies and celiac disease (TEDDY Study Group. 2007). It was important to assess the stool samples from 3-18 months old TEDDY children since immunological regulatory bacteria in the infant microflora may be related to age of onset, rate of progression to disease, or alternatively protection from islet autoimmunity or celiac disease. As an observational cohort study, the TEDDY study currently has more than 6,700 participants who were younger than 4.5 months at the first visit and have HLA genotypes that confer risk for type 1diabetes or celiac disease. The participating clinics have so far collected more than 27,000 stool samples in COL, 14,000 in Georgia/Florida, 25,000 in Washington, 29,000 in FIN, 11,500 in Germany and 49,000 in Sweden to be used to test the above hypotheses. The results of the present investigation suggest that it should be feasible to analyze stool samples from TEDDY children before they developed islet autoimmunity, tissue transglutaminase autoantibodies, or both, to relate content of lactobacilli to the appearance of these disease markers. Alternatively, stools samples from children with these disease markers will be analyzed for lactobacilli associated with progression to clinical onset of either type 1diabetes or celiac disease.

The molecular detection of total lactobacilli content and *L.plantarum* was performed with well-established qPCR methods in an accredited laboratory (Probi AB. Lund, Sweden). It was noticed using the Probe Match program at the RDPII website (http://rdp.cme.msu.edu/) that the primers used to amplify the 16S rRNA gene from *Lactobacillus* would also amplify *Weissella* and *Pediococcus* sequences. However, *Pediococcus* and *Weisella* are uncommon in the human microbiome. The lack of specificity is therefore not likely to complicate the assessment of the number of Lactobacillus in the present investigation.

More importantly, the present results in mail-in stool samples were comparable to previous investigations of more rigorously collected stool samples (Gronlund, et al., 1999, Ahrne, et al., 2005). Traditional plating and culture methods for bacterial detection provided comparable results despite shortcomings in culture techniques such as insufficient selectivity and the presence of non-cultivable bacteria (Nadkarni, et al., 2002).

In a previous study of lactobacilli colonisation in 112

Swedish infants at the age of 3-18 months, the stool samples were collected in controlled conditions, refrigerated and processed within 24 hours. Significant frequency of infants (45%) colonized by lactobacilli was reached at 6 months of age while the highest frequencies were reached before 8 months of age to decline by 12 months of age (Ahrne, et al., 2005). These results are consistent with the present results in mail-in stool samples, where the variability of total lactobacilli were higher in the first 10 months of age to decline and stabilize after 10 months of age in all three clinical sites included in this study. Other studies with variable ways of collecting the stool samples have also shown a large variation in lactobacilli content during the first year of life (Ahrne, et al., 2005, Vael, et al., 2011). It's known that the use of antibiotics decreases the lactobacilli content in stool (Guarner and Malagelada. 2003, Savino, et al., 2011). It remains to be determined whether the marked variation in lactobacilli and *L. plantarum* content within and between individuals may be due to probiotic supplement use or also antibiotic use.

In conclusion, mail-in stool samples from parents participating with their 3-18 months old children made it possible to select significant amounts of DNA whether the stool samples were mailed in COL, FIN or SWE. The higher DNA content in stools mailed in the winter in Finland suggests that season may affect stool sample quality. The similar DNA content in the stool samples in COL, FIN and SWE made it possible to compare clinical sites. The excursions of lactobacilli content in stool before 10 months versus after 10 months of age in both COL and FIN suggest that probiotics may have been used more often, had remained longer, or both, before 10 months of age. Mail-in stool samples from infants should be useful to determine whether the use of probiotics is reflected in the stool and of possible importance to the risk for type 1 diabetes or celiac disease.

ACKNOWLEGMENTS

We thank Josefine Hiller for assistance and the Probi AB, Lund, Sweden for allowing us to use their accredited facilities for lactobacilli PCR analysis.

Funded by DK 63829, 63861, 63821, 63865, 63863, 63836 and 63790 and Contract No. HHSN267200700014C from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institute of Allergy and Infectious Diseases (NIAID), National Institute of Child Health and Human Development (NICHD), National Institute of Environmental Health Sciences (NIEHS), Juvenile Diabetes Research Foundation (JDRF), Centers for Disease Control and Prevention (CDC) and in part by Probi AB, Lund, Sweden.

APPENDIX

The TEDDY Study Group consisted of the following members from 10 centers who served on 15 different committees.

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